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A Comparative Study of the Selective Germicidal Action of Some Common Dyes Upon Bacteria

A COMPARATIVE STUDY OF THE SELECTIVE GERMICIDAL ACTION OF SOME COMMON DYES UPON BACTERIA

BY

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Cecil Robert Gross

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Contents.

Part I. Historical 1

Part II. Experimental 8

Conclusions. 29

Bibliography. 30

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Part I.

Historical.

Early observations by botanists demonstrated that certain anilin dyes were injurious toward plant cells. Cornil and Babes[1], two botanists, showed moreover that methyl violet was toxic toward certain bacteria. Spina (1887)[2] noted that adding a few drops of a saturated aqueous solution of indigo, or of methylene blue, to 100 cc. broth cultures of various organisms resulted in a reduction of the dye. This is one of the first indications of a reaction that is used at the present time in the familiar putrescibility test. The solutions used by Spina were too dilute to manifest any antiseptic action.

Penzoldt (1890)[3] noted a definite bactericidal action of various stains. In this year, Stilling of Strassburg (1890)[4] published a thesis upon this subject. He used a dye, which was probably a mixture of dyes, which he called by the generic name of pyoktannin. His conclusions, based upon none too carefully performed experiments upon B. subtilis, B. anthracis, M. aureus, etc., were that the dye was a general bacterial poison, and that its antiseptic strength was three times that of mercuric bichloride. He considered that the antiseptic strength of the dye was directly proportional to the dilution used, and to the time of exposure; and that the cell membrane played an important part in the process.

Stilling and other investigators made an enthusiastic attempt to introduce the use of the dye as a therapeutic agent.

Extravagant claims were made for it. So little success was attained in the infections upon which it was tried, that the bactericidal action of the dye became seriously questioned. Investigations in this direction were so discouraging that little appeared in the literature during the next ten years.

Interest concerning the effects of dyes upon bacteria was finally aroused in a different connection. Conradi and Drigalski (1902)[5], in searching for a differential medium for the ready isolation and cultivation of B. typhosus, noticed that crystal violet inhibited numerous cocci and bacteria, but had no effect upon B. coli, B. typhosus, and a number of organisms related to B. subtilis. This marks the first hint of a selective action being possessed by a dye.

Investigations soon yielded other effects induced by dyes. Walker and Murray (1904)[6] noticed morphological and biological changes brought about by growing B. typhosus, B. coli, and the cholera vibrio in media containing .2% of a saturated alcoholic solution of methyl violet, gentian violet, fuchsin, methyl green or methylene blue. B. typhosus showed long, actively motile filaments after 5 - 6 hours incubation in gentian violet broth. In older (24 - 48 hour) cultures, segmented portions were visible at one end. In old cultures motility was lost. These results are not convincing in the light of the results of Churchman, which will be referred to later.

Studies following this period have been in the main undertaken along two lines. In one case, the object of investigation has been to find bactericidal qualities which would give to dyes

a valuable application in the treatment of infections. Upon the other hand, the object of experimentation has been to find differential media for the isolation of certain specific bacteria.

Studies upon the bactericidal action of dyes were stimulated by Ehrlich and Shiga (1904)[7], who investigated the trypanocidal action of trypan red. Nicolle and Mesnil (1906)[8], Bouffard (1906)[9], and Goldman (1909)[10] worked upon similar problems, using trypan red and related dyes.

May (1912)[11] concluded from a study of basic fuchsin, that it was a stronger germicide than phenol, more diffusible, less toxic, and that it exerted a stimulating action upon the growth of fresh epithelial and granulation tissue. He hoped to find a use of the dye in the treatment of lupus and blastomycosis. This suggests how Stilling in 1890 advocated the use of anilin dyes in surgical dressings because of their diffusibility, harmlessness, activity upon bacteria, and noncoagulability of any albumen. During this period, one successful differential medium had been devised. Loeffler (1907)[12] discovered that a lactose agar medium containing malachite green and nutrose in the proper proportions would inhibit B. coli, but would allow the growth of members of the paratyphoid group.

The only other indication of a selective bactericidal action being possessed by a dye occurred in the previously cited observation of Conradi and Drigalski (1902)[5], who found that crystal violet inhibited numerous cocci and bacilli, but had no effect upon B. coli, B. typhosus, and a number of organisms re-

lated to B. subtilis. Since their object was a differential medium for the isolation of B. typhosus, they did not follow up this point.

It remained for Churchman (1912)[13] to discover that a dye, gentian violet, exhibited a markedly selective bactericidal action towards bacteria. The growth of Gram positive organisms is usually inhibited by dilutions of gentian violet as high as 1 to 1,000,000. The growth of Gram negative organisms is not interfered with by concentrations of 1 to 5,000 and above. The results were similar whether the bacteria were stained and subsequently transferred at varying intervals to suitable culture media, or whether the dye was added to the medium in or upon which the bacteria were grown. Results were more constant in the second case. Spores were gravely affected by the dye.

Churchman's principal method consisted in the use of a Petri dish divided into two compartments by a thin strip of Halsted's aneurysm metal. One side was poured with plain agar, and then the other side was poured using gentian violet agar. When the media had cooled, the strip was removed. The plate was streaked upon each side from a broth culture, using a fine platinum loop.

Gram positive bacteria failed to develop upon or near the gentian violet agar. Gram negative organisms grew with equal readiness upon the gentian violet agar and the plain agar. Churchman suggests that this selective property may be valuably used in purifying cultures, in devising differential media, or in treating infections.

Later in the same year Churchman and Michael (1912)[14]

noted that 5 strains of B. enteritidis, secured from different sources, were identical in all morphological, tinctoral, and cultural tests, yet exhibited a difference toward gentian violet. The growth of one strain was regularly prevented by the dye so that it could be readily identified from the others. Agglutination tests showed that the gentian violet affinity made as delicate a test as the serum affinity. Further studies may yield valuable methods for differentiation between members of very closely related groups of bacteria such as the B. coli-typhi group, etc.

The selective bactericidal action of gentian violet was found by Churchman (1913)[15] to be shared by dyes chemically related to that dye. The similar action was exhibited by dahlia, parafuchsin, magenta, pararosanalin, rosanalin, crystal violet, and methyl violet 5 B. The selective property is probably due to a common chemical group. Further evidence of this fact is given by methylene blue, a dye unrelated in chemical structure to the former dyes. Churchman (1913)[16] found that this dye exhibited a much less constant selective action upon the gram positive organisms.

Some interesting experiments were carried on by Churchman and Herz (1913)[17] to determine the toxicity of gentian violet upon animals. Injections of 10 cc. of a 1 to 200 aqueous solution of the dye into dogs and rabbits showed gentian violet as a rule to be innocuous in action. The dye rapidly disappears from the system, little or none being left 45 minutes after the injection. The fact that the dye is so readily eliminated from the system would prove important should a therapeutic use be devised.

This series of observations by Churchman stimulated fur-

ther studies of the dyes upon the part of other investigators. Dewitt (1913)[18] showed that methylene blue, bismarck brown, and brilliant cresyl violet are bactericidal in vivo upon Bact. tuberculosis, and that janus green, gentian violet, basic fuchsin, pyronin and neutral red were powerful in vitro, while eosin A and erythrosin had little or no affect. Jansen (1914)[19] concluded from the results of experiments carried on with dyes made up for staining, that all pathogens except spore formers were rendered safe after staining with carbol-fuchsin or anilin gentian violet by the usual laboratory methods, but that staining coverglass smears with methylene blue did not always result in the death of the bacteria. If they survived the process of drying and fixing, they were capable of reproduction after being stained with methylene blue. This is an important consideration to the laboratory student who is staining pathogenic organisms for microscopic examination.

Russell (1914)[20] performed some very interesting experiments with gentian violet upon protozoa. The dye caused them to lose motility, and in some cases to disintegrate. Gentian violet is evidently a true vital stain, as kariokinesis could be observed to go on in a stained nucleus. Embryonic and adult frog tissue grew in vitro in a concentration of 1 to 20,000 of gentian violet. This is a concentration more than sufficient to kill Gram positive bacteria. Selective action upon different tissues was shown by the dye, which prevented the growth of connective tissue in vitro, but allowed the growth of endothelial tissue.

Several new selective media have been devised within the past few years. Torrey (1913)[21] devised a brilliant green dex-

trose broth which surpassed Loeffler's malachite green medium in selective ability toward members of the paratyphoid-enteritidis group of bacteria. Holt-Harris and Teague (1916)[22] found that B. typhosus made a characteristic growth in agar media containing a proper mixture of eosin and methylene blue. The authors were very successful in isolating B. typhosus from scores of stools by means of this medium. Teague and Travis (1916)[23] found that characteristic colonies of B. cholerae would develop in lactose agar containing eosin and bismarck brown.

Leitch (1916)[24] appears to have been successful in the therapeutic treatment of wounds by brilliant green solutions. He finds that it is an active antiseptic towards anaerobes as well as aerobes, and that its use gives speedy relief and usually stimulates the production of granulation tissue. It stains only skin and necrotic tissue, which is a distinct advantage in allowing their identification and removal.

Much investigation upon the effects of dyes upon bacteria remains to be done. Further studies should be warranted by the results already attained. Advances have been made in applying these results in the formulation of successful differential media. There are indications that some of the dyes may find a valuable application in the treatment of infections. Already the use of brilliant green has yielded promising results. Studies of the processes of nuclear development may be facilitated. Further applications may lie in now unsuspected fields.

Part II.

Experimental.

This study was suggested by the investigations of Churchman, whose observations concerning the selective bactericidal action possessed by gentian violet have been referred to in Part I. The extent to which other dyes possessed this property promised an interesting investigation. This study was planned to confirm previous observations and to extend the observations to include a number of common dyes.

The divided plate experiments were first repeated upon a number of Gram positive and Gram negative organisms. The technique used was essentially the same as that employed by Churchman.

Technique of Divided Plates.

An ordinary Petri plate was divided into two compartments by a partition of smooth, glazed, white cardboard. Aneurysm metal strips, as used by Churchman, could not be obtained. The use of cardboard, introducing no injurious substances into the media, seemed preferable to the use of tin, or other metals. The cardboard was cut into strips of .5 cm. width, and about a cm. longer than the diameter of the plate. The strip was cut along a slight curve to conform to the slightly convex bottom of the plate. The ends were bent to leave a length slightly greater than the diameter of the plate, so that when the strip was placed in the plate, it made a firm partition held in place by its elasticity. After sterilization, one side of the plate was poured with gentian violet agar, tilting the plate to allow the agar to approach the partition slowly.

Little would run through under these conditions. When the media had hardened, the partitions were removed with sterile forceps. A stroke of the forceps along the partition severed the agar from it, and allowed its removal together with much or all of the agar that had flowed through clinging to it. The other half of the plate was then poured with 5 cc. of plain agar. This resulted in scarcely an irregularity of surface separating the two media. Churchman's method differs from the one above only in the use of Halsted's aneurysm metal as a partition, and in the removal of the partition after both sides of the plate had been poured.

The gentian violet agar was prepared by adding 1 cc. of a 1 to 100 aqueous solution of gentian violet to 1000 cc. of plain agar made in the usual manner and standardized to a + 10 reaction, according to Fuller's scale. This gave a 1 to 100,000 dilution of gentian violet in plain agar.

Inoculations, as in Churchman's method, were made from broth cultures, using a fine platinum loop to streak the surface of the media. A loopful of broth culture was drawn several times back and forth in the same track across the gentian violet agar. The process was repeated upon the plain agar.

The plates were incubated at 37^0, except in the case of B. prodigiosus, which was grown at 20° C. Examinations for growth were made at the end of 1, 2, 5, 7 and 14 days.

Results of Growth Upon Divided Plates.

The results were in absolute confirmation of Churchman's

observations. A marked selective action was apparent which separated the bacteria into 2 groups. One group of bacteria refused to grow upon the gentian violet agar. These organisms were B. subtilis, B. cereus, B. mesentericus, B. arborescens, B. megatherium, B. butyricus, and B. anthracis. Note that these are all Gram positive bacteria. The other group of bacteria grew as readily upon gentian violet agar as upon plain agar. In this way behaved B. prodigiosus, B. pyocyaneus, B. coli (2 strains), B. proteus vulgaris, B. typhosus, B. paratyphi "A", and B. paratyphi "B", all of which are Gram negative organisms. Gentian violet exhibits an inhibitive action upon Gram positive organisms which it does not possess toward Gram negative organisms.

Not only do Gram positive organisms fail to grow upon gentian violet agar, but they refuse to grow closer than 1 to 2 cm. to it. Churchman noticed the same thing and attributed it to the diffusion of traces of gentian violet into the plain agar. This would indicate that these organisms are extremely sensitive to the dye. An experiment was conducted to determine to what extent the dye might be diluted and yet prevent the growth of Gram positive bacteria.

Quantitative Considerations.

The investigation of the comparative sensitiveness of Gram positive and Gram negative organisms towards gentian violet was conducted upon a number of bacteria from each class. For this purpose, tubes of agar were prepared containing 10 cc. quantities of 8 dilutions of gentian violet ranging from 1 to 10,000 to 1 to 2,000,000. These dilutions were obtained by making preliminary plain

broth dilutions of gentian violet ranging from 1 to 100 to 1 to 200,000 and diluting them 100 times with ordinary plain agar.

Sterilization was carried on in the autoclav for 30 minutes at 10 pounds pressure. These conditions were not found to alter or to precipitate the dye. The tubes were cooled to 45° C., and a set containing one tube of each dilution of gentian violet was inoculated in each case with a small loopful from a broth culture of the organism being studied. A plate was poured from each tube. Observations upon growth in the plates were made at the end of 1, 2, 3, 5, 7 and 12 days' incubation.

The following table (see next page) brings out the main points resulting from this investigation. The comparative sensitiveness of Gram positive and Gram negative organisms towards gentian violet is very pronounced. A mere trace of gentian violet (1 to 1,000,000 to 1 to 2,000,000) is sufficient to prevent the growth of the Gram positive organisms used. This is in harmony with the results of Churchman. Contrast upon the other hand the ability of the Gram negative organisms to develop in a dilution of 1 to 10,000. They manifest, however, a slight sensitiveness toward this concentration which is marked by a less rapid growth and the development of a fewer number of colonies. This influence extends to the 1 to 100,000 dilution in the case of B. prodigiosus. A delay in the production of pigment occurs in these same dilutions.

Colonies of Gram negative organisms developing upon the 1 to 10,000 gentian violet agar plates became deeply stained with the dye. The cumulative influence of the dye might be expected to at least produce a reduction in vitality.

The Growth of Bacteria in Different Concentrations of Gentian Violet in Agar.

Organism	Gram stain	Incubation period (days)	Concentration of Gentian Violet (1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	Plain agar control
B. subtilis	+	1	-	-	-	-	-	-	-	-	-
		3	-	-	-	-	-	-	-	-	+
		12	-	-	-	-	-	-	-	+	++
B. cereus	+	1	-	-	-	-	-	-	-	-	++
		3	-	-	-	-	-	-	-	-	++
		12	-	-	-	-	-	-	-	-	++
B. prodigiosus	-	1	-	++	++	++	++	++	++	++	++
		3	+	++	++	++	++	++	++	++	++
		12	+	++	++	++	+++	++	++	++	++
B. coli	-	1	+	++	++	++	++	++	++	++	++
		3	+	++	++	++	++	++	++	++	++
		12	+	++	++	++	++	++	++	++	++
B. pyocyaneus	-	1	+	++	++	++	++	++	++	++	++
		3	+	++	++	++	++	++	++	++	++
		12	+	++	++	++	++	++	++	++	++
B. capsulatus	-	1	+	++	++	++	++	++	++	++	++
		3	+	++	++	++	++	++	++	++	++
		12	+	++	++	++	++	++	++	++	++

-	indicates no growth, except with reference to Gram stain.
+	indicates a moderate number of colonies, except as above.
++	indicates a large number of colonies.

(1) 1:10,000 (2) 1:100,000 (3) 1:200,000 (4) 1:400,000

(5) 1:600,000 (6) 1:800,000 (7) 1:1,000,000 (8) 1; 2,000,000

Such was not found to be the case. Subcultures from old colonies upon such plates evidenced as unaltered morphological, cultural, and biochemical characteristics as did subcultures from the control plates. The influence of this dilution upon Gram negative organisms is insufficient to produce a more marked effect than a delayed growth.

The sensitiveness of organisms toward fuchsin is not as pronounced as that toward gentian violet. An experiment parallel to the preceding one was conducted upon fuchsin. Growth of these same Gram positive bacteria (B. subtilis and B. cereus) is always prevented by a 1 to 100,000 dilution of fuchsin in agar, is usually prevented by a dilution of 1 to 200,000, but is not prevented by a dilution of 1 to 400,000. The Gram negative organisms grow as readily upon 1 to 10,000 dilutions of fuchsin in agar as in plain agar. Later experiments demonstrated their ability to grow in dilutions of 1 to 5,000. These results indicate that although fuchsin does not possess a quantitative selective ability equal to that of gentian violet, still it shows a marked selective ability and a consequent similarity of action to gentian violet.

These preliminary experiments apparently confirmed the truth of Churchman's earlier observations, so that investigation was begun upon some of the problems which the results suggested. Churchman's observations had shown that the selective bactericidal action of gentian violet was shared by a few dyes related to it in chemical structure. He found this to be true in the case of dahlia, parafuchsin, pararosanalin, rosanalin, crystal violet, and methyl violet 5 B. These results suggested opportunites for

future investigation. Was the selective action of these few dyes shared by a large number of dyes? Was there any correlation between this selective ability and the chemical structure of the dye? These two questions were made the subject of investigation.

Eighteen common dyes were selected from the stock of Grübler dyes. The organic chemist usually divides dyestuffs into 15 groups upon the basis of structural relationships. Sixteen of the 18 dyes fell into 4 of these groups. The following classification shows the chemical relationships of these 16 dyes:

I. Azo-dyestuffs
  a) Chrysoïdine
  b) Orange G.
  c) Bismarck brown
  d) Vesuvine
II. Triphenylmethane dyestuffs
  a) Malachite green
  b) Fuchsin
  c) Gentian violet
  d) Crystal violet
  e) Dahlia
  f) Methyl green
  g) Night blue
III. Xanthene dyestuffs
  a) Eosine A
IV. Quinone - imide dyestuffs
  a) Thionine

b) Methylene blue
c) Neutral red
d) Safranine

Two dyes were used which did not fall into this classification. Orcein is a dihydroximethylbenzene. Picrocarmine consists of a mixture of picric acid with carmine, a red coloring matter isolated from the cochineal insect.

There was a choice of methods by which the inhibitive action of these dyes might be compared. The most direct method would be to incorporate the dyes into the media in which the organisms were cultivated. The divided plate technique of Churchman was considered. This had the disadvantage of quite an outlay of apparatus and time. The presence or absence of growth in media containing the dye could be demonstrated as readily in a broth culture as in an agar plate, and could be more simply accomplished. The method subsequently used consisted in inoculating 1 to 10,000 dye broths with the organisms under observation, incubating for a definite period, and subculturing into plain broth or to agar slants. The absence of growth in a subculture indicated a bactericidal action of the dye in 1 to 10,000 dilution. The comparative bactericidal action of many dyes upon many organisms was easily undertaken.

Technique.

Nutrient broth was prepared in the usual manner and standardized to a +10 reaction according to Fuller's scale. A 1 to 10,000

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

dilution of each dye in nutrient broth was prepared by adding 2 cc. of a 1 to 200 stock aqueous solution of the dye to 100 cc. of nutrient broth. While all the dyes used were readily soluble in water, the stock aqueous solutions of some of the dyes habitually contained a slight sediment. Inaccuracies in the dilution obtained in the dye broth were cut to a minimum by shaking the 1 to 200 solution vigorously and quickly measuring out the required amount into the broth. But slight sediments occurred in the resulting dye broth in the majority of cases. Any decrease in the number of sterilizations given the broth after the addition of the dye decreased the probability of a sediment being formed. Therefore the dye broth was tubed at the time of preparation and sterilized once in the autoclav for 30 minutes at 10 pounds pressure. The following table shows the relative amounts of sediment in the 1 to 200 aqueous stock solution of the dye, in the 1 to 10,000 freshly prepared dye broth, and in the 1 to 10,000 sterilized broth. In all but two cases, the method resulted in a dye broth containing little or no sediment and varying very little from a 1 to 10,000 dilution of the dye. In the case of both vesuvine and night blue, the dye broth prepared by this method contained so much sediment as to appreciably alter the original concentration of these dyes. No satisfactory method was devised to remedy this situation. The effect of these two dyes will therefore not be comparable with the effect of the other dyes upon bacteria.

In order to deal with smaller quantities of media, and to economize space in the incubating room, small tubes of the type used for blood serum cultures were used. Approximately 3 cc. of the dye

Relative Amounts of Sediment.

Dye	1:200 aqueous solution	1:10,000 dye broth (fresh)	1:10,000 dye broth (sterilized)
Picrocarmine	-	-	-
Orceìn	some	-	-
Chrysoïdine	some	-	-
Orange G	-	-	-
Bismarck Brown	some	-	-
Vesuvine	some	much	much
Malachite green	some	v. slight	-
Fuchsin	some	-	v. slight
Crystal violet	v. slight	v. slight	-
Gentian violet	slight	v. slight	-
Dahlia	slight	-	-
Methyl green	-	-	-
Night blue	-	much	v. much
Eosine A	-	-	-
Thionine	some	-	-
Methylene blue	-	-	-
Neutral red	v. slight	v. slight	v. slight
Safranine	v. slight	v. slight	-

Terms range from -, v. slight, slight, some, much, to v. much sediment.

broth was measured into a tube.

The dye broth tubes were in all cases inoculated with a drop of a 2-day broth culture of the organism. This was very conveniently managed by attaching a rubber tube and pinchcock to a sterile pipette held in a nearly vertical position by a clamp. The pipette could be sucked nearly full of a culture, and a series of tubes could be very rapidly inoculated. As the mouthpiece of the pipette as well as about an inch of the rubber tubing was always kept plugged with cotton, no danger of contamination from this source was incurred. Contaminations were never experienced by the use of this method.

The comparative germicidal action of the dyes was observed upon 35 different bacteria comprising 30 definite species. The choice was made from common pathogenic and non-pathogenic organisms which grew readily upon artificial media. An endeavor was made to secure cultures from reliable sources. Most of the organisms used were subcultures of strains obtained from the collection in the American Museum of Natural History in New York City. Other determinations upon their authenticity or truth to type were not made.

Incubation was carried on at 37° C. B. prodigiosus and S. ventriculi were incubated at 20° C. An incubation period of 5 days gave the most reliable results. The results given later are based upon this period of incubation. A shorter period of incubation produced conflicting data, the reason for this being discussed later. A longer period of incubation was inadvisable, as evaporation might be expected to cause a greater con-

centration of the dye accompanied by an increase of bactericidal action.

At the end of the incubation period, a subculture was made from each dye broth culture into plain broth tubes containing at least 5 cc. of plain broth. The small amount of dye carried over in the platinum loop became diluted beyond the possibility of causing any antiseptic action. Sterility of such a subculture would indicate that the organism had been killed by an exposure of 5 days or less to the action of a 1 to 10,000 solution of the dye. More than an antiseptic action is felt because the dye broth was inoculated with a drop of a 2 day broth culture of the organism, which would introduce thousands of bacteria into 3 cc. of dye broth. Many bacteria would certainly be transferred in inoculating from the dye broth, and some of these should develop in the subculture. It is possible that the organism had been so severely injured as to prevent multiplication. Such a condition must be very close to death, as these organisms were not revived by transplanting to favorable media. The intensity of action of these dyes depends upon the concentration and the duration of application. This has repeatedly been determined. If the injury is so close to death upon exposure to a 1 to 10,000 dilution for 5 days or less, the concentration or the duration of application would need be little increased to result in death. We can therefore conclude with practical certainty that sterility in these subcultures is due to a germicidal action of the dye.

Results.

The results from the comparative study of the germicidal action

Table I.

Gram Positive Bacteria.

Species	Picrocarmine	Orcein	Chrysoidine	Orange G	Bismarck Brown	Vesuvine	Eosine	Thionine	Methylene blue	Neutral red	Safranine	Malachite green	Fuchsin	Dahlia	Crystal violet	Gentian violet	Methyl green	Night blue	Plain agar
B. mesentericus	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	+	+
B. subtilis	+	+	-	+	+	+	+	-	-	+	-	-	-	-	-	-	-	+	+
B. megatherium	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	+	+
B. cereus	+	+	-	+	+	+	+	-	-	+	-	-	-	-	-	-	-	+	+
B. arborescens	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+
B. butyricus	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+
B. aurantiacus	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+
M. ureae	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	+	+
M. tetragenus	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+
Staph. albus	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+
B. granulosum	+	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+
S. lutea	+	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+
S. ventriculi	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
B. smegma	+	+	-	+	±	+	+	-	±	-	±	±	-	±	±	±	-	+	+

Table II.

Gram Negative Bacteria.

Species	Picrocarmine	Orcèin	Chrysoïdine	Orange G	Bismarck brown	Vesuvine	Eosine	Thionine	Methylene blue	Neutral red	Safranine	Malachite green	Fuchsin	Dahlia	Crystal violet	Gentian violet	Methyl green	Night blue	Plain agar
B. cloacae	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. aerogenes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. coli communis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. coli communior	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+
B. coli communior #1	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
B. coli acidi lactici	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. alkaligenes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. typhi #1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. typhi #2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	+	+	+
B. typhi #3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. typhi #607	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. paratyphi "A"	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. paratyphi "B"	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. enteritidis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. fluorescens #9	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
B. fluorescens #2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. capsulatus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. pyocyaneus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. prodigiosus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. proteus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. violaceus	+	+	-	+	+	+	+	-	-	+	-	-	-	-	-	-	-	+	+

of dyes upon bacteria are incorporated in Table I and II. The Gram positive organisms are grouped together in Table I, the Gram negative in Table II. A comparison of the two tables brings out that there are a number of dyes which are germicidal with regularity towards Gram positive bacteria, but possess this ability upon Gram negative organisms in only a very few isolated cases. The 11 dyes possessing to a greater or less extent this selective germicidal power are methyl green, gentian violet, crystal violet, dahlia, fuchsin, malachite green, safranine, neutral red, methylene blue, thionine and chrysoïdine. Churchman has previously determined that 5 of these dyes, gentian violet, crystal violet, dahlia, fuchsin and methylene blue possess a greater or less degree of selective bactericidal action . The selective bactericidal action of the remaining six, so far as I know, has not previously been reported.

The extent to which vesuvin and night blue may possess bactericidal qualities can not be determined from these tables. Their consideration may be dismissed with the previously made statement that no method could be devised to obtain homogeneous solutions of these dyes in broth, consequently rendering the results incomparable with those of other dyes. There is an indication that they may possess a greater bactericidal power than the results indicate, in that the 1 to 200 aqueous stock solution of each is sterile.

The 5 dyes remaining for discussion exhibited practically no bactericidal action. These dyes are picrocarmine, orcèin, orange G, bismarck brown and eosine. Two of these dyes, picrocarmine and orange G, showed no germicidal action upon any of the organisms used, Gram positive or Gram negative. The remaining 3 dyes showed no bactericidal

action upon any of the Gram negative bacteria, nor upon most of the Gram positive bacteria, only inhibiting the three least hardy organisms in this very susceptible class.

The sensitiveness of bacteria to the bactericidal action of the dyes is primarily a characteristic of the Gram positive bacteria. The Gram negative organisms almost without exception are quite immune to this action. The exception is B. violaceus. This organism behaved toward the dyes exactly like the Gram positive organisms. It is considered to be a Gram negative organism by most writers, and this strain was found to be undeniably Gram negative. Outside of this case the results show a distinct correlation between the bactericidal action of the dyes and the Gram stain.

Constancy of Results.

The failure of any organism to grow in any of the germicidal dyes was very constant and could be repeatedly demonstrated. The main exception is B. smegma. This organism was inhibited with irregularity by the same dye (see Table I). No explanation can at present be offered except that this action may have some correlation with the acid fast character of this organism toward dyes.

The remaining cases were confined to the early experiments and among the Gram negative organisms, where sometimes an organism might give an uncertain growth in the presence of a particular dye. It was noticed that these conflictioning results occurred in

a dye broth culture subcultured after a period of 24 to 48 hours' incubation. A dye broth culture subcultured after a longer period of incubation gave either a uniformly positive or a uniformly negative growth. Uniform results could be obtained in an incubation period of 5 days.

This fact was attributed to a condition similar to the germicidal phase in the development of microorganisms in milk. It has been pointed out previously in this thesis that less numerous and more slowly developing colonies resulted in agar plates containing a 1 to 10,000 or a 1 to 100,000 dilution of gentian violet than in a plate containing a 1 to 2,000,000 dilution, or in a control plate, all equally inoculated. Even the comparatively unsusceptible Gram negative organisms find the greater concentrations slightly antiseptic, or perhaps slightly germicidal. So it is reasonable to suppose that a subculture taken from a dye broth culture after a short period of incubation might be taken at the time there were the fewest living or multiplying organisms present, so that the loop might or might not carry over living organisms.

Differentiation Between Closely Related Strains.

The selective bactericidal action is so definite in action that it might very well constitute a convenient basis of differentiation between different strains of bacteria in closely related groups. Churchman(15) was able to differentiate one strain of B. enteritidis from 4 others, because it regularly failed to grow upon gentian violet agar. Attention has been called to the

regular failure of some Gram negative organisms to grow in the presence of one or another of the dyes. A comparative study of the selective bactericidal action of these dyes upon closely related strains might yield certain combinations which would distinguish between these strains.

A comparative study of this nature was made upon members of the colon - typhoid - enteritidis group, and lately, upon members of the B. proteus group, in which the data is not yet complete. The 11 dyes which had shown the greatest bactericidal action upon the Gram positive organisms were used. The bacteria were inoculated into plain broth containing a 1 to 10,000 dilution of the dye, and the bactericidal action was determined, as in the preceding experiment, by subculturing after a period of 5 days' incubation.

The negative growth shown by subcultures (see Table III) were not as numerous as had been hoped. The only cases lie in the dahlia, crystal violet and gentian violet columns. They do not occur in a great enough variety of cases to admit the separation of all the members of a group from one another by any combination, altho one strain may be separated from the others of a group. The three B. coli communior cultures would admit of the following separation:

	Dahlia	Crystal violet
B. coli communior	-	-
B. coli communior #1	-	+
B. coli communior #2	+	+

These results are promising. It is possible that increasing the concentration of the dye in broth may result in a greater number of negative growths. This is a good subject for future experimen-

tation.

Table III.

Members of the Colon-Typhoid-Enteritidis Group.	Chrysoïdine	Thionine	Methylene blue	Neutral red	Safranine	Malachite green	Fuchsin	Dahlia	Crystal violet	Gentian violet	Methyl green	Plain agar
Colon Strains												
B. coli communis	+	+	+	+	+	+	+	+	+	+	+	+
B. coli communior	+	+	+	+	+	+	+	-	-	+	+	+
B. coli communior #1	+	+	+	+	+	+	+	-	+	+	+	+
B. coli communior #2	+	+	+	+	+	+	+	+	+	+	+	+
B. coli acidi lactici	+	+	+	+	+	+	+	+	+	+	+	+
Typhoid Strains												
B. typhi #1	+	+	+	+	+	+	+	+	+	+	+	+
B. typhi #2	+	+	+	+	+	+	+	+	±	+	+	+
B. typhi #3	+	+	+	+	+	+	+	+	+	+	+	+
B. typhi #4	+	+	+	+	+	+	+	+	+	+	+	+
B. typhi #5	+	+	+	+	+	+	+	+	+	+	+	+
B. typhi #6	+	+	+	+	+	+	+	+	-	+	+	+
B. typhi #607	+	+	+	+	+	+	+	+	+	+	+	+
Paratyphoid Strains												
B. paratyphi "A" #1	+	+	+	+	+	+	+	+	+	+	+	+
B. paratyphi "A" #177	+	+	+	+	+	+	+	+	-	+	+	+
B. paratyphi "A" #247	+	+	+	+	+	+	+	+	+	+	+	+
B. paratyphi "B" #1	+	+	+	+	+	+	+	+	+	+	+	+
B. paratyphi "B" #16	+	+	+	+	+	+	+	+	-	+	+	+
B. paratyphi "B" #600	+	+	+	+	+	+	+	+	+	+	+	+
Enteritidis Strains												
B. enteritidis (Gaertner)	+	+	+	+	+	+	+	+	+	+	+	+

+ indicates growth

- indicates no growth.

Correlation between Germicidal Action and Chemical Structure of the Dye.

Upon the basis of germicidal ability, the dyes fall into two groups. One group manifests a distinctly selective germicidal action upon the Gram positive bacteria, a character not possessed by the other group of dyes. When the dyes (see Table IV) are grouped according to their structure, there is noted a tendency for the actively germicidal dyes to lie in certain groups. Every member studied in both the triphenylmethane group and the quinone-imide group are "active". The other groups are made of up of "inactive" dyes with the exception of chrysoïdine, lying in the azo-group. An endeavor was made to determine whether the "active" dyes did not contain a common bond which was lacking in the structure of the inactive dyes, but no definite conclusion was reached after a most careful study of their structural formulae. The investigation must be extended to other groups of dyes, and further data obtained before this point can be settled.

Table IV.

Classification of Dyes.	Selective Germicidal Ability
I. Hydroxi - benzene derivatives	
a) methyl - 1. Picrocarmine	-
b) nitro - 1. Orcèin	-
II. Azo - dyestuffs	
a) monazo - 1. Chrysoidine	+
2. Orange G	-
b) diazo - 1. Bismarck brown	-

		Selective Germicidal Ability
III. Triphenylmethane dyestuffs		
a) dibasic -	1. Malachite green	+
b) tribasic -	1. Fuchsin	+
	2. Dahlia	+
	3. Crystal violet	+
	4. Gentian violet	+
	5. Methyl green	+
IV. Xanthene dyestuffs		
	1. Eosine A	-
V. Quinone - imide dyestuffs		
a) Thiazines-	1. Thionine	+
	2. Methylene blue	+
b) Azines -	1. Neutral red	+
c) Safranines-	1. Safranine	+

Conclusions.

The selective bactericidal action previously observed by Churchman(13),(15),(16) to be possessed by a few aniline dyes, is a chracteristic common to at least 11 aniline dyes, chrysoïdine, thionine, methylene blue, neutral red, safranine, malachite green, fuchsin, dahlia, crystal violet, gentian violet and methyl green.

These dyes possess a germicidal action toward Gram positive bacteria and a negative action toward Gram negative bacteria thru a wide range of concentrations. The Gram negative bacteria are not absolutely immune, for their growth is prevented in certain concentrations. They are, however, very much less sensitive to the dyes than are the Gram negative organisms.

The selective germicidal action is of so constant and specific a nature that it would constitute as definite a biochemical characteristic as any used to identify bacteria if it were not for the uncertain behavior of a few organisms.

The specific ability of a dye to prevent the growth of certain Gram negative organisms may admit of certain combinations of the dyes which would distinguish between closely related strains of Gram negative organisms. This point presents an opportunity for future investigation.

No bactericidal ability is possessed by at least 5 anilin dyes, picrocarmine, orcèin, orange G, bismarck brown, and eosine.

The uniform selective germicidal ability of dyes in 2 well defined chemical groups of dyes, and the almost universal absence of this quality displayed by dyes in 3 other chemical groups suggests that the action is due to a common chemical bond, which

a careful comparison of the structural formulae of the dyes failed, however, to identify. An investigation into the selective germicidal action of dyes of widely diverse structure might solve this point.

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